REMARKS

Applicants thank the Examiner for his Advisory Action and for considering the Exhibits Applicants filed with a Supplemental Response to the Final Office Action.

In contrast to the Examiner's comments, there is no evidence that the RIPA lysates used in Exhibits A and B are different. In fact, both antibodies were analyzed with EGF-treated A431 carcinoma cells. Thus, the differences in the antibodies is due solely to their different reactivities. That is, not all anti-phosphotyrosine antibodies react with every phosphorylated tyrosine establishing that anti-phosphotyrosine antibodies do not bind to every phosphorylated tyrosine of every protein. As stated in our previous response, it is well known that anti-phosphotyrosine antibodies have specificity and selectivity. Thus, for instance, attached as Exhibits A and B are copies of certificates of analysis of two different anti-phosphotyrosine antibodies sold by Upstate, the cited 4G10 antibody and a rabbit polyclonal anti-phosphotyrosine antibody mixture. These certificates of analysis show that under the same conditions, different proteins were recognized by the different antibodies. In turn, these certificates of analysis show that not every tyrosine phosphorylated antibody will recognize the same proteins, i.e., the same phosphorylated tyrosines.

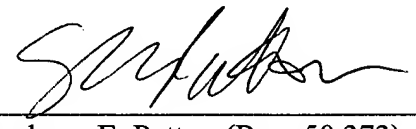
In view of such evidence, it can not be assumed or even expected that antibodies reported by Buday and Hirth would "specifically bind to a LAT polypeptide comprising an amino acid sequence according to SEQ ID NO: 4."

It is believed the application is in conditions for immediate allowance, which action is earnestly solicited.

A separate Petition for Extension of Time is also submitted herewith for a one (1) month

extension of time to May 13, 2005. Applicant also conditionally petitions for a further extension time to provide for the possibility that such a petition is required. Please charge Deposit Account No. **04-1105** for the required fee.

Respectfully submitted,



Date: June 13, 2005

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Certificate of Analysis

Anti-Phosphotyrosine
(rabbit immunoaffinity purified IgG)
Catalog # 06-427
Lot # 25163

Immunogens: In order to produce broad spectrum polyclonal phosphotyrosine antibodies, rabbits were immunized with three phosphorylated immunogens: (1) phosphotyrosine covalently linked to KLH; (2) the c-Src carboxyl terminal regulatory phosphopeptide (T-S-T-E-P-Q-pY-Q-P-G-E-N-L; Catalog # 12-218) covalently linked to KLH, and; (3) a phosphopeptide associated with high tyrosine kinase activity in human lymphocytes (R-R-L-I-E-D-A-E-pY-A-A-R-G; Catalog # 12-217) covalently linked to KLH. Both of the phosphopeptide haptens serve as strong substrates for tyrosine phosphatases and are part of the two colorimetric tyrosine phosphatase kits provided by Upstate, Inc. (Catalog # 17-125, 17-126).

Species Cross-reactivity: Human, mouse and rat. Other species cross-reactivity is unknown.

Specificity and Purification: The immunoreactivity of the antibody is totally inhibited by the use of 100mM phenyl phosphate, a phosphotyrosine analog. The phosphotyrosine antibody is purified by immunoaffinity chromatography using either a dual phospho-peptide gel or a BSA-phosphotyrosine gel. All of the phosphotyrosine immunoreactivity present in the antisera is immunoadsorbed whether the antibody is purified by either gel indicating that the antibody is not sequence-specific but specific for phosphotyrosine residues.

Formulation: **200µg** of immunoaffinity purified rabbit IgG in **800µl** of 0.2M Tris-glycine, pH 7.2, 0.15M NaCl, 5mg/ml of BSA containing 0.05% sodium azide. Concentration: 0.25mg/ml. Frozen solution.

Storage and Stability: Stable for 1 year at -20°C from date of shipment. Aliquot to avoid repeated freezing and thawing. For maximum recovery of the product, centrifuge the original vial after thawing and prior to removing the cap.

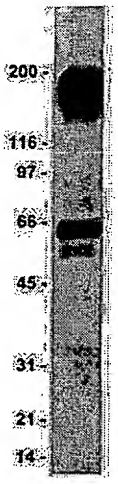
FOR RESEARCH USE ONLY
NOT FOR USE IN HUMANS

Quality Control Testing

Immunoblot Analysis: 0.5-2µg/ml of this lot detected proteins containing phosphotyrosine residues in RIPA lysates from EGF-stimulated human A431 carcinoma cells.

Included Positive Antigen Control: Catalog # 12-302, EGF-stimulated A431 cell lysate. **Add 2.5µl of 2-mercaptoethanol/100µl of lysate and boil for 5 minutes to reduce the preparation.** Load 20µg of reduced lysate per lane for minigels.

Immunoprecipitation: 4µg of this lot immunoprecipitated proteins containing phosphotyrosine residues from a human A431 RIPA lysate.



Immunoblot Analysis
Representative blot from a previous lot. EGF-stimulatedA431 cell lysate was resolved by electrophoresis, transferred to nitrocellulose and probed with anti-Phosphotyrosine (0.5µg/ml). Proteins were visualized using a goat anti-rabbit secondary antibody conjugated to HRP and a chemiluminescence detection system.

References:

1. The cSrc carboxyl terminal regulatory phosphopeptide (T-S-T-E-P-Q-pY-Q-P-G-E-N-L) which binds to the internal SH2 domain of c-Src.
   Song, Z., *et al.*, Cell **72:**767, 1993.
   Luttrel, D.K., *et al.*, Proc. Natl. Acad. Sci. USA **91:** 83, 1994.
2. The phosphopeptide associated with high tyrosine kinase activity in human lymphocytes (R-R-L-I-E-D-A-E-pY-A A-R-G).
   Trevillyan, J.M., *et al.*, Biochim. Biophys. Acta **845**: 1, 1985.

Immunoblot Protocol

1. Perform SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on a cell lysate sample (cell lysis buffer: 50mM Tris-HCl, pH 7.4; 1% NP-40; 0.25% sodium deoxycholate; 150mM NaCl; 1mM EGTA; 1mM PMSF; 1μg/ml aprotinin, leupeptin, pepstatin; 1mM Na_3VO_4; 1mM NaF) and transfer the proteins to nitrocellulose. Wash the blotted nitrocellulose twice with water.
2. Block the blotted nitrocellulose in freshly prepared PBS containing 3% nonfat dry milk (Catalog # 20-200) and 0.05% Tween-20 (PBST-MLK) for 20 minutes at room temperature with constant agitation.
3. Incubate the nitrocellulose with **0.5-2μg/ml of anti-Phosphotyrosine**, diluted in freshly prepared PBST-MLK, overnight with agitation at 4°C.
4. Wash the nitrocellulose twice with water.
5. Incubate the nitrocellulose in the secondary reagent of choice (a **goat anti-rabbit** HRP conjugated IgG, Catalog # 12-348, 1:5000 dilution, was used) in PBST-MLK for 1.5 hours at room temperature with agitation.
6. Wash the nitrocellulose with water twice.
7. Wash the nitrocellulose in PBS-0.05% Tween 20 for 3-5 minutes.
8. Rinse the nitrocellulose in 4-5 changes of water.
9. Use detection method of choice (enhanced chemiluminescence was used).

Immunoprecipitation Protocol

1. Add **4μg of anti-Phosphotyrosine** and 60μl (30μl packed beads) of washed Protein A agarose bead slurry (Catalog # 16-125) to 500μl of PBS in a microcentrifuge tube.
2. Gently rock the reaction mixture at 4°C for 1 hour.
3. Collect the agarose beads by pulsing (5 seconds in the microcentrifuge at 14,000 x g), and drain off the supernatant. Wash the beads 3 times with either ice-cold cell lysis buffer or PBS.
4. Dilute the cell lysate to roughly 1μg/μl total cell protein with PBS.
5. Add 500μg-1mg cell lysate to the reaction mixture.
6. Gently rock the reaction mixture at 4°C for 1 hour.
7. Collect the agarose beads by pulsing (5 seconds in the microcentrifuge at 14,000 x g), and drain off the supernatant. Wash the beads 3 times with either ice-cold cell lysis buffer or PBS.
8. Resuspend the agarose beads in 60μl 2X Laemmli sample buffer.
9. Store the beads frozen for future analysis or boil the beads for 5 minutes.
10. Collect the beads after boiling using a microcentrifuge pulse.
11. Perform SDS-PAGE and immunoblot analysis on a sample of the supernatant fraction.

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Certificate of Analysis

Anti-Phosphotyrosine, clone 4G10

(mouse monoclonal $IgG_{2b\kappa}$)
Catalog # 05-321
Lot # 26349

Immunogen: Phosphotyramine-KLH.

Antibody Class: $IgG_{2b\kappa}$ mouse monoclonal antibody produced *in vitro* by mouse-mouse hybridoma 4G10 (FOX-NY [NS-1 derivative] myeloma x spleen cells). Purified by Protein G-Sepharose chromatography.

Formulation: **100µg** of protein G purified mouse monoclonal $IgG_{2b\kappa}$ in **100µl** of 0.1M Tris-glycine, pH 7.4, 0.15M NaCl, 0.05% sodium azide. Liquid at 4°C.

Storage and Stability: Stable for 2 years at 4°C from date of shipment. **NOTE: DO NOT FREEZE.** For maximum recovery of the product, centrifuge the original vial prior to removing the cap. If the product has accidentally been frozen and thawed, spin it at 13,000 x g for 10 minutes at 4°C. Save the supernatant for application.

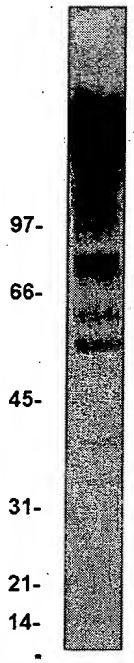
FOR RESEARCH USE ONLY
NOT FOR USE IN HUMANS

Quality Control Testing

Immunoblot Analysis: 0.5-2µg/ml of this lot detected tyrosine-phosphorylated proteins in a modified RIPA lysate from EGF-treated human A431 carcinoma cells.[1,2,3]

Included Positive Antigen Control: Catalog # 12-302, EGF-stimulated A431 cell lysate is provided as a free positive antigen control for western immunoblotting. Aliquot as desired, refreeze immediately, and store at -20°C. The lysate is stable for 6 months at -20°C. Before use, **add 2.5µl of 2-mercaptoethanol/100µl of lysate and boil for 5 minutes to reduce the preparation.** Load 20µg of reduced lysate per lane for immunoblot analysis.

Immunoprecipitation: 2-4µg of this lot can immunoprecipitate quantitatively the phosphotyrosine-containing proteins in the lysate of a confluent culture (10cm dish) of cells expressing an activated tyrosine kinase. To preserve phosphotyrosine, add 0.2mM sodium orthovanadate to the lysis buffer.



Immunoblot Analysis:
Representative blot from a previous lot. EGF-stimulated A431 cell lysate was resolved by electrophoresis, transferred to nitrocellulose and probed with anti-phosphotyrosine (1µg/ml). Proteins were visualized using a goat anti-mouse secondary antibody conjugated to HRP and a chemiluminescence detection system.

References:
1. Cohen, B., *et al.*, Proc. Natl. Acad. Sci. USA. **87**: 4458-4462, 1990.
2. Druker, B.J., *et al.*, New Eng. J. Med. **321:** 1383-1391, 1989.
3. Kanakura, Y., *et al.*, J. Biol. Chem. **266:** 490-495, 1991.

Immunoprecipitation Protocol

1. Add **2-4µg of anti-Phosphotyrosine, clone 4G10** and 60µl (30µl packed beads) of washed Protein A agarose bead slurry (Catalog # 16-125) to 500µl of PBS in a microcentrifuge tube.
2. Gently rock the reaction mixture at 4°C for 1 hour.
3. Collect the agarose beads by pulsing (5 seconds in the microcentrifuge at 14,000 x g), and drain off the supernatant. Wash the beads 3 times with either ice-cold cell lysis buffer or PBS.
4. Dilute the cell lysate to roughly 1µg/µl total cell protein with PBS.
5. Add 500µg-1mg cell lysate to the reaction mixture.
6. Gently rock the reaction mixture at 4°C for 1 hour.
7. Collect the agarose beads by pulsing (5 seconds in the microcentrifuge at 14,000 x g), and drain off the supernatant. Wash the beads 3 times with either ice-cold cell lysis buffer or PBS.
8. Resuspend the agarose beads in 60µl 2X Laemmli sample buffer.
9. Store the beads frozen for future analysis or boil the beads for 5 minutes.
10. Collect the beads after boiling using a microcentrifuge pulse.
11. Perform SDS-PAGE and immunoblot analysis on a sample of the supernatant fraction.

Immunoblot Protocol

1. Perform SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on a cell lysate sample (cell lysis buffer: 50mM Tris-HCl, pH 7.4; 1% NP-40; 0.25% sodium deoxycholate; 150mM NaCl; 1mM EDTA; 1mM PMSF; 1µg/ml aprotinin, leupeptin, pepstatin; 1mM Na_3VO_4; 1mM NaF) and transfer the proteins to nitrocellulose. Wash the blotted nitrocellulose twice with water.
2. Block the blotted nitrocellulose in freshly prepared PBS containing 3% nonfat dry milk (Catalog # 20-200), (PBS-MLK) for 45-90 minutes at room temperature with constant agitation.
3. Incubate the nitrocellulose with **0.5-2µg/ml of anti-Phosphotyrosine, clone 4G10,** diluted in freshly prepared PBS-MLK overnight with agitation at 4°C.
4. Wash the nitrocellulose twice with water.
5. Incubate the nitrocellulose in the secondary reagent of choice (a goat anti-mouse HRP conjugated, Catalog # 12-349, 1:2000 dilution, was used) in PBS-MLK for 1.5 hours at room temperature with agitation.
6. Wash the nitrocellulose with water twice.
7. Wash the nitrocellulose in PBS-0.05% Tween 20 for 3-5 minutes.
8. Rinse the nitrocellulose in 4-5 changes of water.
9. Use detection method of choice (enhanced chemiluminescence with a 30 second exposure was used).